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CAT VENTRICULAR MUSCLE TREATED WITH D600: CHARACTERISTICS OF CALCIUM CHANNEL BLOCK AND UNBLOCK

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SUMMARY

1. Thin preparations of cat ventricular muscle were mounted in a single sucrose gap and superfused with Tyrode solution containing 1–5 μM-D600. In voltage-clamp experiments lasting for 40–180 min, stimulation with standard pulses (−50 to 0 mV, 300 ms) at 0·33 Hz depressed Ca-dependent slow inward current (I_{Ca}) to less than 20 % of its pre-drug amplitude.

2. A reproducible unblocking of *ca.* 75 % of the blocked Ca channels could be achieved with a single hyperpolarizing pulse (90 s at −90 mV); stimulation (conditioning) at 0·33 Hz re-established full block within thirty pulses. The time and voltage dependence of block and unblock were examined by varying the frequency and duration of voltage-clamp pulses.

3. The time course of unblock was usually monoexponential. The time constant was voltage dependent and declined from 9 min at −50 mV to 5 s at −110 mV.

4. Block appears to depend on channel state, resting channels being highly resistant to block and open channels very susceptible. D600 also binds to inactivated channels but at a much slower rate than to open channels.

5. A small U-shaped component of block was induced by conditioning to potentials between +10 and +80 mV. This block seemed to be unrelated to channel state, suggesting that drug binding may also be dependent on voltage.

6. Quicker rates of block after repetitive conditioning, and slow wash-out of the drug, may indicate the existence of an intramembrane drug pool distinct from the primary pool in the intracellular fluid.

7. The interaction of D600 with Ca channels is discussed in terms of a channel state model. In many respects this interaction resembles that of local anaesthetics with Na channels.

INTRODUCTION

Electrophysiological experiments on cardiac preparations treated with local anaesthetic–antiarrhythmic agents provided the earliest indications that a block of ionic channels by drug molecules can be more complex than a simple plugging of channel pores. Weidmann (1955) employed the $\dot{V}_{max}$ of the action potential upstroke

as an index of Na current (I_{Na}) and found that its depression by quinidine could be relieved by hyperpolarization. His conclusion was that quinidine blocks I_{Na} by shifting the steady-state inactivation curve to more negative potentials. An additional signal of voltage involvement was the enhanced depression of $\dot{V}_{max}$ by antiarrhythmics in KCl-depolarized preparations (Watanabe, Dreifus & Likoff, 1963; Singh & Vaughan Williams, 1971; Chen, Gettes & Katzung, 1975). Stimulus frequency also emerged as an important factor when it was established that the drug-induced depression of $\dot{V}_{max}$ is more severe at high driving rates than at low ones (Johnson & McKinnon, 1957; Heistracher, 1971; Tritthart, Fleckenstein & Fleckenstein, 1971).

Insight into these results has evolved from studies with local anaesthetics on frog node of Ranvier (Strichartz, 1973; Courtney, 1975; Khodorov, Shishkova, Peganov & Revenko, 1976; Hille, 1977), squid axon (Narahashi, Frazier & Moore, 1972; Cahalan, 1978; Cahalan & Almers, 1979; Yeh, 1979) and skeletal muscle fibres (Schwarz, Palade & Hille, 1977). In brief, charged drug molecules inside the cell can most easily reach the Na channel receptor when the channel gates are open. Drug binding is therefore enhanced, the more often channels are opened by depolarizing pulses (i.e. voltage-dependent, frequency-dependent, use-dependent or conditioned block). Block itself may be due to physical obstruction of ion flow by the drug molecule, and/or to an interaction of the drug with the inactivation gating mechanism. The latter may shift steady-state inactivation such that large hyperpolarizations are required for the removal of inactivation. While the opening of the h gates with hyperpolarization allows drug to escape from the channel, the rate of drug binding appears to be much greater when both the h and the m^3 gates are opened.

D600, verapamil and AQA39 are closely allied compounds which depress Ca-dependent slow inward current (I_{Ca}) in the heart (Kohlhardt, Bauer, Krause & Fleckenstein, 1972; Kass & Tsien, 1975; Nawrath, Ten Eick, McDonald & Trautwein, 1977; Trautwein, Pelzer, McDonald & Osterrieder, 1981; Osterrieder, Pelzer, Yang & Trautwein, 1981). Stimulus frequency and voltage are also important variables in the action of these drugs. Expressions of this include the enhanced depression of nodal transmission, action potential plateau and force of contraction as the stimulation rate is increased (Wit & Cranefield, 1974; McCans, Lindenmayer, Munson, Evans & Schwartz, 1974; Bayer, Kalusche, Kaufmann & Mannhold, 1975), and the enhanced depression of automaticity in isolated sinus node preparations that are partially depolarized (Osterrieder *et al.* 1981).

Voltage-clamp studies have shown that the block of Ca channels in cardiac tissue by verapamil (Ehara & Kaufmann, 1978), D600 (McDonald, Pelzer & Trautwein, 1980, 1984) or AQA39 (Osterrieder *et al.* 1981; Trautwein *et al.* 1981; Pelzer, Trautwein & McDonald, 1982) is voltage dependent: hyperpolarizing pre-pulses relieve the channel block induced by repetitive depolarizing pulses. These studies have also provided an explanation for the frequency-dependent effects of these drugs: the depression of I_{Ca} is enhanced by an increase in the frequency of depolarizing pulses. In addition, it has been shown that D890, the permanently charged quaternary derivative of D600, is effective on intracellular but not extracellular application (Hescheler, Pelzer, Trube a Trautwein, 1982).

In this study we have examined some of the factors pertinent to the block and unblock of the Ca channels in cat ventricular muscle treated with D600. Preliminary

results were communicated to the International Congress of Pharmacology, Tokyo, in 1981 (Trautwein, Pelzer & McDonald, 1983) and additional aspects of D600 action on ventricular muscle are presented in the preceding paper (McDonald *et al.* 1984).

METHODS

Papillary muscles or ventricular trabeculae of diameter 0·15–0·4 mm and length 3–5 mm were isolated from cat heart and mounted in a single sucrose gap for voltage-clamp experiments. Complete details of the experimental arrangement are described in the preceding paper (McDonald *et al.* 1984). The Tyrode solution perfusing the right (test) compartment of the sucrose gap contained (in mM): NaCl, 140; KCl, 5·4; $MgCl_2$, 1·0; $CaCl_2$, 1·8; $NaHCO_3$, 12·0; NaH_2PO_4, 0·4; glucose, 5·0. The solution in the middle (sucrose) compartment contained 304 mM-sucrose (enzymic grade), 5 mM-glucose and 0·01 mM-$CaCl_2$ dissolved in de-ionized water. In the Tyrode solution perfusing the left (KCl) compartment, NaCl was replaced by KCl. Tyrode solutions were equilibrated with 95 % O_2 and 5 % CO_2 while sucrose solutions were gassed with 100 % O_2. The temperature of the solutions was 36 ± 1 °C and the pH $7{\cdot}4 \pm 0{\cdot}05$. The Tyrode solutions containing D600 at desired concentrations were prepared by adding appropriate amounts of drug stock solution to the normal Tyrode. Tetrodotoxin (Sigma, St Louis, U.S.A.) at 5×10^{-5} M was added to the solutions during the voltage-clamp experiments; this had no detectable effect on the amplitude or time course of I_{Ca}. Unless otherwise indicated, muscles were driven or pulsed at 0·33 Hz.

D600 was kindly provided by Knoll AG, Ludwigshafen, F.R.G.

RESULTS

General features of Ca channel block and unblock

Useful information on Ca channel block by D600 can be obtained from changes in the magnitude of I_{Ca} when activating pulses are preceded by hyperpolarizing pre-pulses of variable duration. The voltage-clamp programme for an experiment of this type is described in Fig. 1*A*. With the holding potential set at −50 mV, activating pulses of 50 mV amplitude and 300 ms duration were preceded by pre-pulses of duration t to potential V_{pp}. Following a period of 0·33 Hz pulsing with no pre-pulse, a pre-pulse to a V_{pp} of −55 mV for 3 s was introduced for the next eight pulses. After changing to pre-pulse durations of 10, 50 and 0·6 s for eight pulses each, the entire sequence was repeated with V_{pp} at −80 mV. The amplitude of I_{Ca} on the eighth pulse at each condition is plotted in Fig. 1*C*. In the absence of D600 (control), a shortening of the pre-pulse duration from 50 to 0·6 s induced a 10–15 % decline in I_{Ca}, and this response was independent of the pre-pulse potential. The picture was quite different after 60–70 min in D600 (2 μM). With V_{pp} at 80 mV, I_{Ca} was about 70 % of the control when the pre-pulse duration was 50 s but less than 30 % of control when the duration was 0·6 s. At the more positive V_{pp} of −55 mV, there was a sharp reduction in I_{Ca} and a flatter relation between current amplitude and pre-pulse duration. The modulation of drug action by pre-pulse voltage is highlighted by the records in Fig. 1*B*. With the pre-pulse duration constant at 50 s, changing V_{pp} from −80 to −55 mV had negligible effect in the pre-drug trial but reduced I_{Ca} by about 75 % after drug treatment.

The results presented in Fig. 1 indicate that the fraction of Ca channels blocked by D600 is dependent on the voltage pattern imposed on the muscle. Frequent depolarization, as occurred with the shorter pre-pulse wave forms, favours channel block; long pre-pulses to negative potentials favour channel unblock. Thus, it is

feasible to study the removal of drug from channel binding sites by applying pre-pulses after block has been imposed with frequent stimulation, and to study the association of drug with the channel by applying various wave forms after effective unblock with pre-pulses.

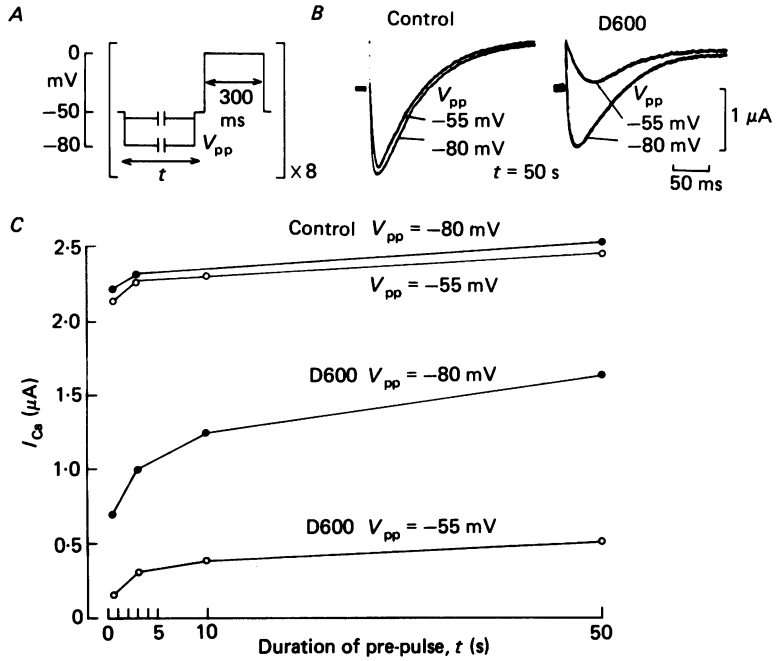

Fig. 1. Dependence of the D600 depression of I_{Ca} on pre-pulse duration and potential. *A*, procedure. Muscles were stimulated with 300 ms pulses to 0 mV from the holding potential of −50 mV. After thirty pulses at 0·33 Hz, a pre-pulse to −55 or −80 mV (V_{pp}) of duration *t* was included for eight pulses. The interval at −50 mV between pre-pulse and test pulse was 0·3 s. *B*, membrane currents on the eighth activating pulse of trains with V_{pp} at −80 or −55 mV for a duration of 50 s. The block of I_{Ca} by D600 (2 μM for 60–70 min) was markedly affected by the pre-pulse potential. *C*, the amplitude of I_{Ca} (eighth pulse) *versus* pre-pulse duration at V_{pp} of −80 and −55 mV. Control *versus* 2 μM-D600 for 60–70 min. The order of the changes in pre-pulse duration was 3, 10, 50 and 0·6 s at a V_{pp} of −55 mV; a similar pattern followed at a V_{pp} of −80 mV.

With the exception of two series of experiments requiring special protocols, all experiments related to block and unblock were conducted on muscles exposed to D600 for 40–180 min. The majority were performed during the 60–100 min period, by which time I_{Ca} with regular stimulation (holding potential −50 mV, 300 ms pulses to 0 mV at 0·33 Hz) had been reduced to 10–20 % of control. In these experiments a standard clamp sequence for effective unblock was adopted: 90 s at −90 mV followed by 10–60 s at −50 mV (see below). In seven muscles treated with D600 for 40–60 min, I_{Ca} on a test pulse subsequent to this unblocking sequence averaged 85 % of the control (pre-drug) I_{Ca} measured with a similar protocol. After 100 min or longer with the drug, the recovery averaged 82 %. The 15–20 % channel block remaining after effective unblock can be termed the tonic (resting, basal) block, and the difference between this and full block with stimulation will be called the conditioned block.

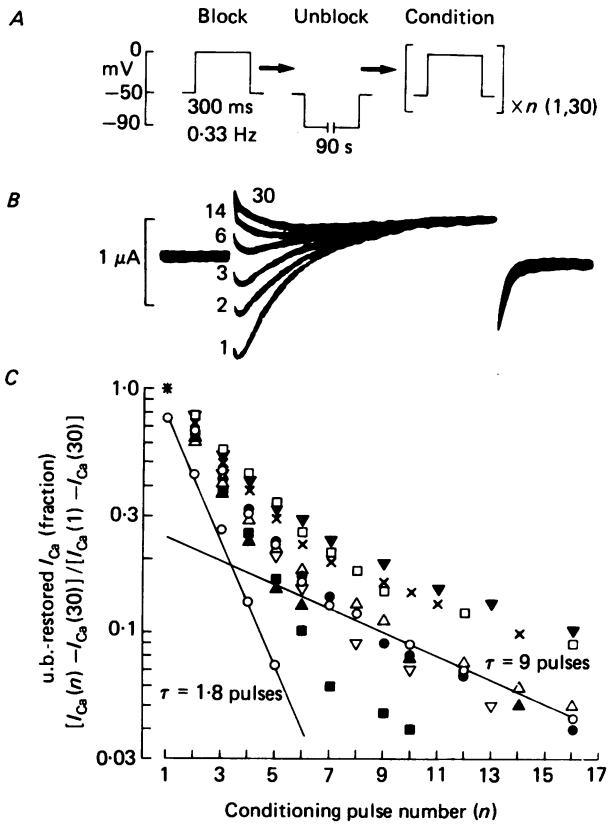

Fig. 2. The time course of the conditioned block of I_{Ca} in muscles treated with 2 μM-D600 for 40–150 min. *A*, procedure. From the holding potential of −50 mV, an unblocking sequence (90 s at −90 mV) was interposed during regular stimulation (300 ms steps to 0 mV at 0·33 Hz). A further 20 s equilibration period at −50 mV was followed by thirty conditioning pulses (300 ms to 0 mV at 0·33 Hz). *B*, membrane currents recorded during a conditioned block. Traces identified by pulse number (n) show that 'steady-state' block is reached within thirty pulses. The lengthening of the time to peak negative current was pronounced on the later conditioning pulses. *C*, time courses of the conditioned block in nine muscles. I_{Ca} restored by the unblocking sequence (u.b.-restored I_{Ca}) was maximal on the first pulse after unblock ($I_{Ca}(1) - I_{Ca}(30)$). The fraction of u.b.-restored I_{Ca} remaining after n conditioning pulses was taken as $[I_{Ca}(n) - I_{Ca}(30)]/[I_{Ca}(1) - I_{Ca}(30)]$; this fraction is plotted on a semilogarithmic scale against the number of conditioning pulses (n). The time course of the conditioned block always had two phases. As an example, the block in one muscle (○) is described as the sum of two exponentials with time constants of 1·8 and 9 pulses.

The time course of the conditioned block was determined in nine muscles treated with 2 μM-D600 for 40–150 min (Fig. 2). In each case, block under regular stimulation (300 ms pulses from −50 to 0 mV at 0·33 Hz) was relieved with the standard unblocking sequence (90 s at −90 mV). The resumption of stimulation 20 s later produced a pulse to pulse accumulation of block, I_{Ca} being depressed to its previous blocked level within thirty pulses (Fig. 2*B*). I_{Ca} restored by the unblocking sequence, u.b.-restored I_{Ca}, was taken as the difference in amplitude between I_{Ca} triggered by

a given pulse (n) in the train, and I_{Ca} at the full block (i.e. $I_{Ca}(n) - I_{Ca}(30)$). The u.b.-restored I_{Ca} was maximal on the first conditioning pulse, and the fraction of u.b.-restored I_{Ca} remaining after n conditioning pulses was calculated with reference to this maximum (i.e. $[I_{Ca}(n) - I_{Ca}(30)]/[I_{Ca}(1) - I_{Ca}(30)]$). When plotted on a semi-logarithmic scale against pulse number, n (Fig. 2*C*), the decay of u.b.-restored I_{Ca} (i.e. the onset of block) occurred in two phases: a large fast one (mean amplitude 0·74; mean time constant, τ, 1·9 pulses) and a small slow one (mean amplitude 0·26; mean τ 9·2 pulses). This is illustrated by the exponentials fitted to values from one of the muscles (○) in Fig. 2*C*.

Methodological problems. The first methodological problem concerns the measurements of I_{Ca} amplitude (taken as the difference between the peak inward current and the late current at 250 ms depolarization). During this time there is partial activation of outward K current, I_K ($\tau \sim 300$ ms at 0 mV), and this results in I_{Ca} being over-estimated by about 10% (McDonald & Trautwein, 1978*a*). However, there is an additional complication. The block of I_{Ca} by D600 prolongs the time to peak of the inward current and this can be explained by the overlap of a transient outward current, I_{to} (McDonald *et al.* 1984). The amplitude and time course of I_{to} are most easily visualized in records taken at full D600 block. The subtraction of I_K from the net current isolates an I_{to} that activates rapidly, inactivates with $\tau \sim 20$ ms, and has an amplitude of 10–15% control I_{Ca}. The maximum amplitude of I_{to} under control conditions was placed at 10–20% of control I_{Ca} amplitude. Therefore, if D600 does block I_{to}, the block is of the same degree as the block of I_K (up to 30%) by the drug (see McDonald *et al.* 1984).

If the foregoing is correct, we can estimate how the presence of I_{to} has affected the measurements of I_{Ca} in unblock–block experiments. (a) Control I_{Ca} was probably underestimated by up to 10%, i.e. underestimate due to maximum I_{to} minus over-estimate due to I_K. (b) Unblocked I_{Ca} has been underestimated by about the same amount if the partial block of I_{to} by D600 was relieved by the unblocking sequence. If there was no removal of I_{to} block, the underestimate was less. (c) Fully blocked I_{Ca} was over-estimated by 5–10% since it is likely that I_{Ca} was fully blocked by D600 (see McDonald *et al.* 1984) and our measurement of I_{Ca} (late current minus inward peak) was always greater than zero. In summary, since unblocked I_{Ca} is likely to have been slightly underestimated, and blocked I_{Ca} to have been slightly over-estimated, the extent and time courses of the blocks are unlikely to be seriously in error.

The second point concerns the unblocking clamp sequences. These included a re-equilibration period at −50 mV to alleviate complications which can arise from long depolarizations or hyperpolarizations, e.g. alterations in the conductance state of ionic channels, and the accumulation or depletion of extracellular K (McDonald & Trautwein, 1978*b*). Depending on the duration of the unblocking pulse, the equilibrium periods at the holding potential ranged from 2 to 60 s. Except where noted in the text, these periods should have had little effect on the measurements in question.

In the sections which follow, we examine the time and voltage dependence of unblock and then concentrate on factors affecting the conditioned block.

The time and voltage dependence of unblock

Fig. 3*A* shows that when the duration of the unblocking pulse is held constant, the restoration of I_{Ca} is dependent on the voltage of the unblocking pulse (V_{ub}). In this example, the duration was set at 20 s and V_{ub} was −50, −70, −80 or −90 mV. Currents triggered by the test pulses after the unblock indicate that the recovery of I_{Ca} improved as V_{ub} was moved in the hyperpolarizing direction. On the other hand, when V_{ub} was held constant at −90 mV, the restoration of I_{Ca} was dependent on the duration of the unblocking pulse (Fig. 3*B*): restoration was negligible for the 1 s pulse, about 40% for the 5 s pulse, and nearly complete for the 60 s pulse.

The time and voltage dependence of the removal of block were examined in

experiments on twelve muscles. In each case the amplitude of I_{Ca} restored by an unblocking pulse, I_{Ca}(test)$-I_{Ca}$(30), was compared with the reference I_{Ca} restored by a 90 s, −90 mV unblocking sequence, i.e. I_{Ca}(ref)$-I_{Ca}$(30) (Fig. 4*A*). The pooled results of the twelve experiments are presented in Fig. 4*B* where the fraction of I_{Ca} restored by unblock is plotted against the duration of the unblocking pulse. There was complete recovery at potentials more negative than −50 mV, the rate of recovery being quicker the more negative the unblocking voltage. At −50 mV, the recovery was incomplete (0·7–0·8 of reference) even after 15 min, the longest time tested. At −20 and −30 mV, recovery was negligible after unblocking pulses lasting 11–14 min.

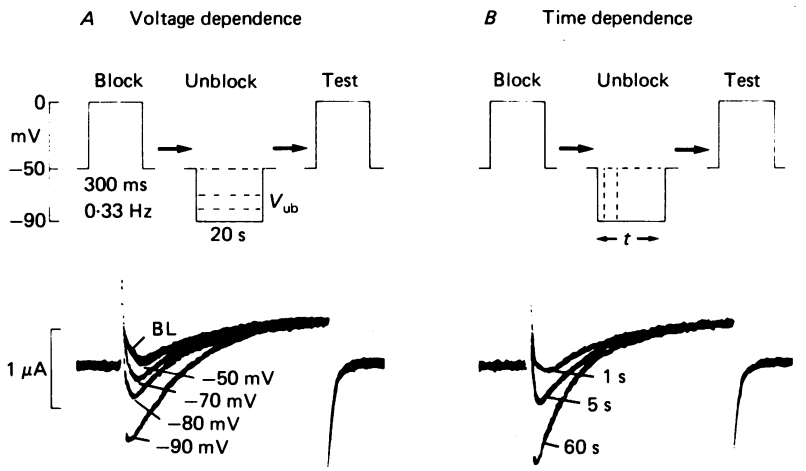

Fig. 3. The removal of Ca channel block depends on the voltage and duration of the unblocking pulse. *A*, voltage dependence. I_{Ca} was blocked (record BL) after 45 min stimulation (−50 to 0 mV for 300 ms at 0·33 Hz) in the presence of 2 μM-D600. An unblocking sequence of constant duration (20 s) to a voltage V_{ub} was followed 10 s later by a standard test pulse. Full block was imposed before each V_{ub} trial. The membrane currents on the test pulses show that unblock increased as V_{ub} was moved to more negative potentials. *B*, time dependence (5 min after the experiment in *A*). Here, V_{ub} was fixed at −90 mV and the duration of the unblocking pulse (t) was varied on each trial. Test pulse currents show that unblock increased with t.

The time courses of removal of D600 block were obtained at V_{ub} values of −50, −70, −90 and −110 mV in four muscles. A semilogarithmic plot of the data from one of these muscles is shown in Fig. 4*C*. The restoration of drug-blocked channels can be described by single exponentials at −50, −90 and −110 mV, but two exponentials are required to fit the unblock at −70 mV. The time constants (mean, range) in the four muscles were (5 s, 3–7 s) at −110 mV, (17 s, 10–23 s) at −90 mV, and (9 min, 6–15 min) at −50 mV. The slow phase of recovery at −70 mV had an average time constant of 2·3 min and average fractional amplitude of 0·87; the small fast phase appeared to have a time constant of about 6 s. Two additional points about unblock deserve mention: (a) unblock at −140 mV seemed to be faster than unblock at −110 mV (partial data only), and (b) it is not proven that full unblock can be achieved at −50 mV; the assumption in Fig. 4*C* is that full unblock occurs when there is a sufficiently long rest period.

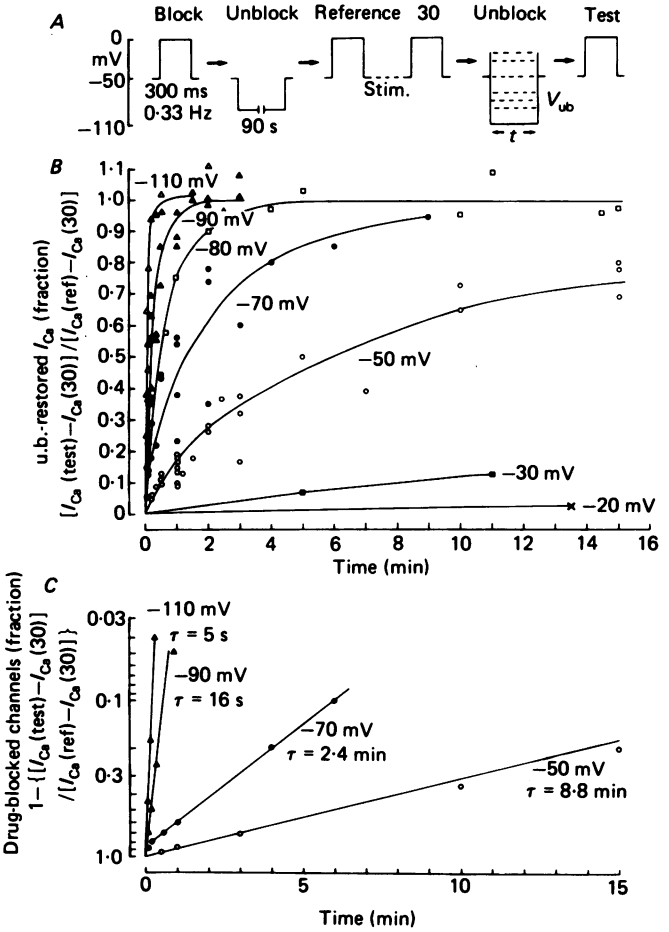

Fig. 4. Dependence of the recovery from conditioned block on time and membrane potential. *A*, procedure. Regular stimulation (300 ms pulses from −50 to 0 mV at 0·33 Hz) was interrupted for an unblocking sequence (90 s at −90 mV, 10 s at −50 mV) and a reference test pulse. Block was then re-imposed with thirty pulses at 0·33 Hz stimulation ('30'). The subsequent unblocking pulse (to potential V_{ub} for duration t) was followed after 10 s at −50 mV by a standard test pulse. (In practice, an unblock reference sequence was usually applied before and after a series of tests rather than before each individual test.) *B*, pooled data from twelve muscles. I_{Ca} restored by an unblocking pulse (u.b.-restored I_{Ca}) was normalized with respect to I_{Ca} restored by the reference unblock, i.e. $[I_{Ca}(test)-I_{Ca}(30)]/[I_{Ca}(ref)-I_{Ca}(30)]$. Curves fitted by eye. Symbols: △, −110 mV; ▲, −90 mV; □, −80 mV; ●, −70 mV; ○, −50 mV; ■, −30 mV; ×, −20 mV. *C*, semilogarithmic plot of the time course of recovery from block in a muscle treated with 2 μM-D600 (70–170 min). Unblock appears to follow a monoexponential course except at −70 mV, and is about 100 times faster at −110 mV than at −50 mV.

Steady-state inactivation. In cat ventricular muscle the steady-state inactivation variable, f_∞, declines in sigmoidal fashion from about 1·0 at −50 mV to 0 at 0 mV (Trautwein, McDonald & Tripathi, 1975). A drug-induced negative shift in f_∞ might account for some of the observations in Figs. 1–4. However, the simplest case, that the presence of drug in the tissue causes a shift in f_∞, can be ruled out by the results

in Fig. 3. Since the unblocking hyperpolarizations were followed by 10 s rests at −50 mV, steady-state inactivation relieved by the hyperpolarization ought to have been re-imposed during the 10 s at −50 mV. This was not the case because initial activating pulses from −50 mV after effective unblock triggered I_{Ca} transients whose magnitudes approached pre-drug values.

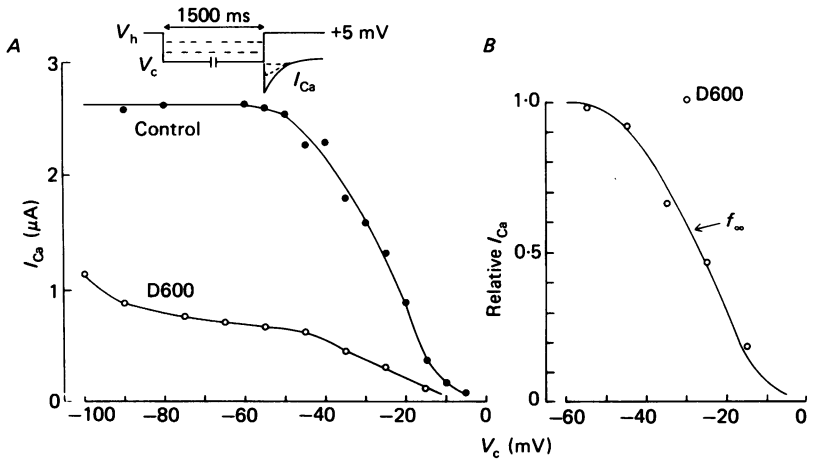

Fig. 5. Determination of the steady-state inactivation (f_∞) relation before and after treatment of a muscle with 1 μM-D600 for 30 min. Following regular stimulation at 0·33 Hz, the holding potential (V_h) was moved to +5 mV. Hyperpolarizing pulses of 1500 ms duration were then applied at 0·33 Hz to potentials V_c (see schematic diagram). Depolarization back to the holding potential triggered I_{Ca} whose amplitude is plotted against V_c in graph *A*. *B*, normalization of control data in graph *A* gives an estimate of f_∞ (continuous curve). When the D600 values negative to $V_c = -60$ mV are ignored, and the remainder normalized with respect to $V_c = -60$ mV, the normalized values (○) fall along the f_∞ curve.

Steady-state inactivation relations were determined in a papillary muscle before and after the application of 1 μM-D600 for 30 min. As shown in the schematic diagram of Fig. 5*A*, the holding potential was set at +5 mV and 1500 ms long hyperpolarizations to potential V_c were applied at 0·33 Hz. Thus, Ca channels inactivated at +5 mV were reprimed at V_c, and the degree of repriming was measured by the relative amplitude of I_{Ca} upon returning the potential to +5 mV. The duration of the repriming pulse was set at 1500 ms to allow (under normal conditions) for complete restoration of I_{Ca} over this voltage range (Trautwein *et al.* 1975). When current amplitude was plotted against the repriming potential (Fig. 5*A*), the pre-drug curve had the usual S-shape but the D600 curve did not saturate at more negative potentials. A plausible explanation for the latter is that we are dealing with two populations of channels: drug-bound and drug-free. The 1500 ms repriming pulses produced negligible unblock at potentials positive to −60 mV but progressively larger unblock as the potential was moved in the negative direction. On this basis we assumed that $I_{Ca}(V_c = -60\text{ mV})$ represented the maximum available current through drug-free conducting channels. Normalization of the values at more positive V_c (open circles, Fig. 5*B*), and their superimposition on the control f_∞ curve, provide

convincing evidence that f_∞ is not shifted in drug-free channels. Whether f_∞ is shifted in drug-bound channels is much more difficult to ascertain (see Discussion).

Unblock with 'cycling'. Strichartz (1973) reported that the voltage-dependent block of Na channels in frog node perfused with QX-314 could be relieved in two ways; slowly with rest alone, or rapidly with repetitive short depolarizations thought to cycle drug-bound channels through the open state. Strichartz emphasized that these depolarizations should be frequent (1–4 Hz), and to a potential where m^3 gates can open but where Na channel block sensitive to conditioning voltage is not too strong. In addition, the relief from block was enhanced when these depolarizations were coupled with hyperpolarizing pre-pulses to potentials where h_∞ approaches unity. Courtney (1975) and Yeh (1979) had similar experiences in their respective studies with GEA-968 and 9-aminoacridine.

To reproduce these conditions in D600-treated ventricular muscle, the holding potential was set at −50 mV and trains of short (20–30 ms) activating pulses to −25 mV were applied, i.e. steady-state inactivation near unity at −50 mV (Fig. 5), and channel opening with relatively weak voltage-dependent block at −25 mV (see Fig. 8). After establishing a block with regular 0·33 Hz for pulses for 1 min, the short pulses were applied at 1–2 Hz for 1 min. Fractional unblock was then assessed with a 300 ms test pulse to 0 mV. The control was a 1 min rest at −50 mV, and the average results of two trials in each of three muscles were (cycling *versus* rest); 0·14 and 0·11, 0·23 and 0·18, and 0·17 and 0·16, respectively. Thus, unblock via cycling with this particular protocol was only marginally more effective than rest alone.

The dependence of block on channel state

Some of the foregoing experiments hint at a dependence of drug–channel interaction on the conductance state of the channel: depolarization (open channels?) promotes block whereas hyperpolarization (inactivated or resting channels?) promotes unblock. In this section we describe experiments which explore in more detail the association of D600 with channels in the resting, open or inactivated state.

Block of resting channels. The experiment depicted in Fig. 6 (schematic diagram) was designed to measure the association of D600 with resting channels. A muscle superfused with control solution was rested for 30 min at −60 mV, and then stimulated at 0·33 Hz with 300 ms pulses to 0 mV. A similar rest–stimulation cycle was then imposed but this time in the presence of 2 μM-D600. The superimposed membrane current records in Fig. 6 show that I_{Ca} on the first post-rest activation in the presence of D600 (D1) was nearly identical to I_{Ca} on the corresponding control activation (C1). However, after an additional 2 min stimulation, I_{Ca} in the presence of the drug (D40) was only one-third as large as control I_{Ca} (C40).

In three additional experiments where the holding potentials during the rest were between −55 and −80 mV, I_{Ca} amplitudes on the C1 and D1 pulses differed by less than 10%. Thus, I_{Ca} channels in the resting state are very resistant to block by D600.

Block of open channels. To examine the association of drug with open channels, we varied the number of openings per unit time, the fraction of channels opened, and the duration of the open time.

In the first of these experiments a muscle treated with 2 μM-D600 for 70 min was

sequentially blocked, unblocked and conditioned (Fig. 7*A*). The amplitude (−50 to 0 mV), duration (300 ms) and number (40) of conditioning pulses were the same in all trials but the pulsing rate was either 0·1, 0·33 or 1 Hz. The plot of I_{Ca} *versus* conditioning time shows that the rate of block increased with the rate of pulsing (Fig. 7*B*). However, when the data are analysed as a function of conditioning pulse number, it is clear that the rate dependence comes from a direct relation between channel block and number of openings (Fig. 7*C*). A further conclusion is that the repriming of unblocked channels at −50 mV is complete within 700 ms – about the time expected for full repriming in untreated muscle (Trautwein *et al.* 1975). If repriming were several times slower in drug-treated muscle (Kohlhardt, Kübler & Herdey, 1974; Kohlhardt & Mnich, 1978), the curve relating block to pulse number at 1 Hz conditioning would have fallen far below the 0·33 and 0·1 Hz curves.

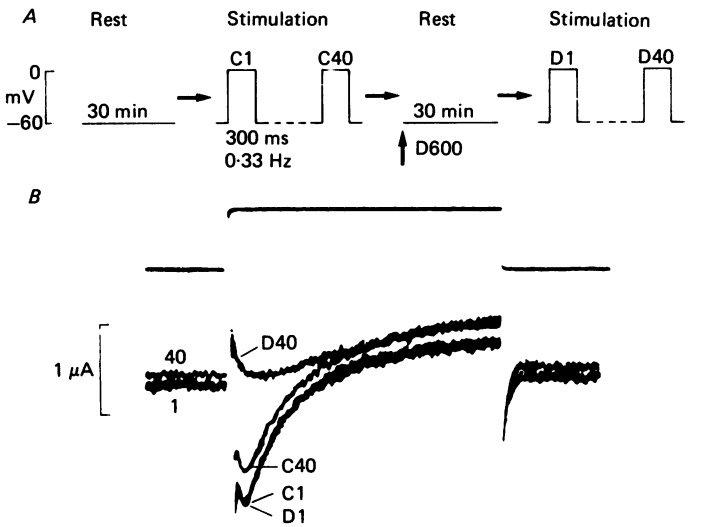

Fig. 6. D600 does not block Ca channels in resting muscle clamped at −60 mV. *A*, procedure. In the pre-drug control voltage-clamp sequence, the muscle was rested at −60 mV for 30 min and then stimulated for 2 min with 300 ms pulses to 0 mV at 0·33 Hz (pulses C1–C40). The rest–stimulation cycle was then repeated but the solution was changed to one containing 2 μM-D600 at the beginning of the rest period. *B*, membrane currents accompanying the first (C1, D1) and fortieth (C40, D40) post-rest pulses before and during D600. Under control conditions the prolonged rest resulted in an increase in I_{Ca} and a reduction in the outward current at −60 and 0 mV, i.e. the C40 membrane current was similar to the control pre-rest membrane current during 0·33 Hz stimulation. D600 had little effect on the membrane current accompanying the first post-rest pulse (D1) but block of Ca channels was very evident by the fortieth post-rest pulse (D40).

The next approach was to measure the dependence of block on the fraction of Ca channels activated by conditioning pulses, the number of activations per unit time remaining constant. The steady-state activation variable, d_∞, is a sigmoidal function of voltage, approaching 0 at −50 mV, 0·5 at −20 mV and 1·0 at +10 mV in cat ventricular preparations (Trautwein *et al.* 1975; Reuter & Scholz, 1977). The normal method of estimating the activation curve involves the application of short (20–30 ms) depolarizing pulses to potentials between −50 and +50 mV. We adopted a similar

protocol to estimate the dependence of block on activation. In three muscles treated with 2 μM-D600 for 60–90 min, block under regular stimulation was followed by a standard unblocking sequence, fifteen 30 ms conditioning pulses at 0·33 Hz to potentials V_c, and a 300 ms test pulse to 0 mV (Fig. 8*A*). As shown by the symbols in Fig. 8*B*, the degree of block increased with the conditioning potential, V_c. For V_c between −50 and +10 mV, the dependence had a sigmoidal shape and closely resembled the d_∞ relation (continuous curve, Fig. 8*B*). However, in contrast to the saturation of d_∞, there was a creep in the block at potentials positive to +10 mV. This finding is examined more closely in a subsequent section.

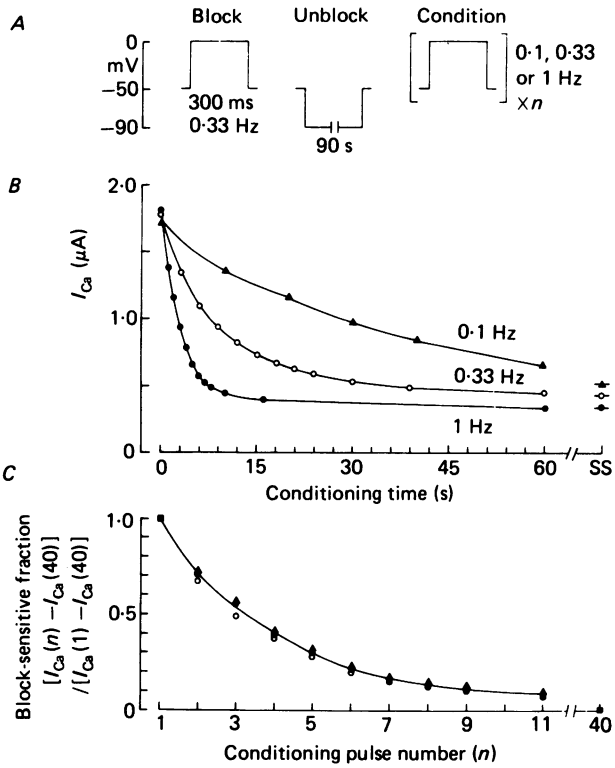

Fig. 7. The dependence of the time course and degree of conditioned block on the rate of stimulation. *A*, procedure. Steady-state block with 300 ms pulses from −50 to 0 mV at 0·33 Hz was followed by unblock and 300 ms conditioning pulses delivered at 0·1, 0·33 or 1 Hz. *B*, decline of I_{Ca} during conditioning in muscle treated with 2 μM-D600 for 70–90 min. 'Steady-state' block (SS) was reached within the forty pulses at each pulsing rate. *C*, decay of the current carried by Ca channels sensitive to block. When plotted against pulse number, the rate of decay is nearly independent of stimulation rate between 0·1 and 1 Hz.

An additional way of varying the 'openness' of channels is to vary the duration of the activating pulse. Since the time constant of Ca channel inactivation (τ_f) in cat myocardium is about 70 ms at 0 mV (McDonald & Trautwein, 1978*a*), the time available for the drug to interact with open channels will be far less with 30 ms pulses than with, for example, 300 ms ones. An indication that openness is a factor in block

is provided by the results in Fig. 8*B* where fifteen 30 ms pulses to 0 mV produced only 70% of the reference 300 ms pulse block.

The experiment depicted in the schematic diagram of Fig. 9*A* was designed to investigate whether block is related to the duration of the conditioning pulse in a simple direct way, or in a way consistent with the availability of open Ca channels.

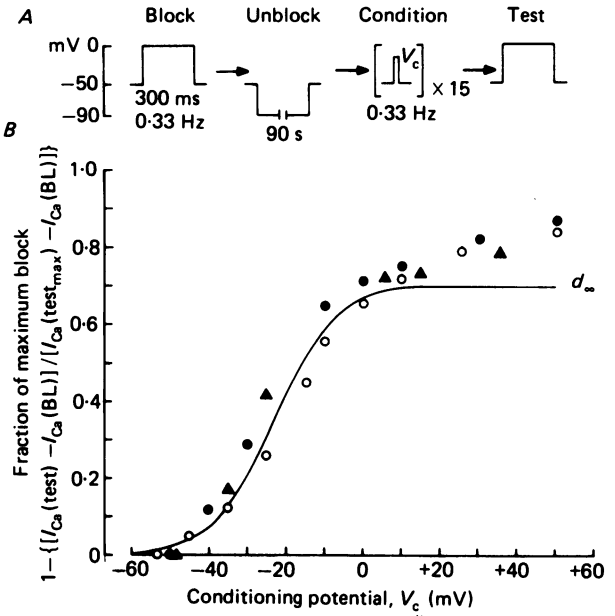

Fig. 8. The relation between block, conditioning potential, and the degree of activation of the Ca system. *A*, procedure. Muscles treated with 2 μM-D600 for 60–90 min were stimulated at 0·33 Hz with 300 ms pulses from −50 to 0 mV (block). Following unblock (90 s at −90 mV, 20 s at −50 mV), they were pulsed fifteen times at 0·33 Hz with 30 ms steps to potentials V_c and then tested for block with a 300 ms pulse to 0 mV. *B*, the fraction of maximum block *versus* the potential of the conditioning pulses (V_c). Maximum block refers to block achieved with the 300 ms pulses to 0 mV at 0·33 Hz. The effect of the conditioning pulses was determined by relating the amplitude of block-sensitive I_{Ca} after conditioning (I_{Ca}(test)−I_{Ca}(block)) to maximum block-sensitive I_{Ca} (I_{Ca}(test$_{max}$)−I_{Ca}(block)); 'test$_{max}$' was measured on test pulses immediately after unblock. Symbols represent determinations on three muscles. The continuous curve is the steady-state activation variable (d_∞) taken from Trautwein *et al.* (1975) and scaled in amplitude to fit the data between −50 and −10 mV. There is a noticeable divergence of the data from d_∞ at potentials positive to 0 mV.

Block during 2 μM-D600 treatment was followed by unblock, conditioning to +5 mV at 0·33 Hz, and a standard test pulse from −50 to +5 mV. The number of conditioning pulses (n) varied from one to sixteen, and the duration of the pulses (t) ranged from 30 to 2400 ms. The dependence of [I_{Ca}(test)−I_{Ca}(block)] on the number of conditioning pulses is plotted in Fig. 9*B*. Block was pulse dependent at each pulse duration but the blocking effectiveness of pulses increased with pulse duration. One conditioning pulse lasting 2400 ms produced as much block as four 300 ms pulses, eight 100 ms pulses or (extrapolated) twenty 30 ms pulses.

A different perspective on the relation between pulse duration and block emerges

when I_{Ca} is plotted against cumulative time at the conditioning potential ($t \cdot n$). Fig. 9*C* indicates that the rate of block slows as the pulse is lengthened. The results with 30, 100 and 300 ms pulses are not out of line with the supposition that block is in some way related to the product of time and the fraction of open channels. It is less clear whether a similar explanation can account for the slow increase in block

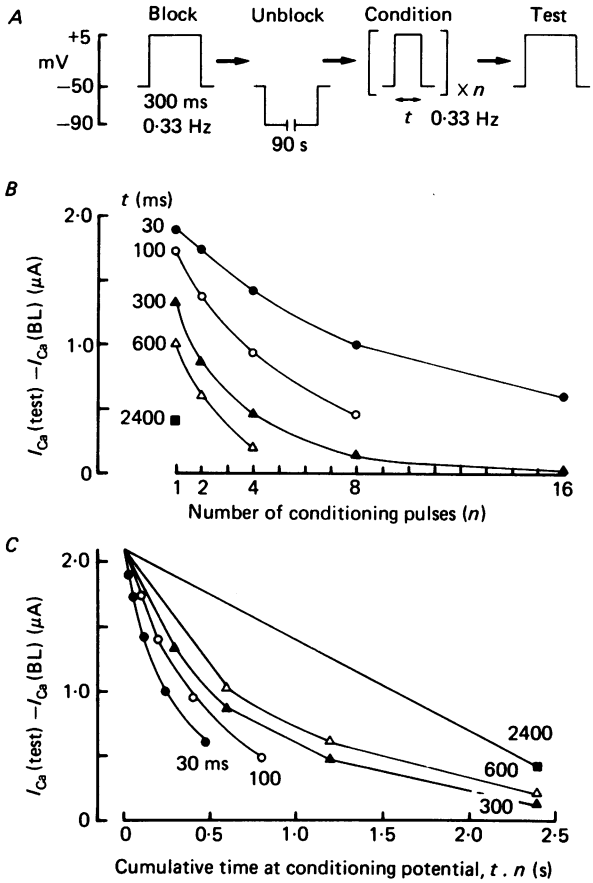

Fig. 9. Dependence of Ca channel block on the duration of the conditioning pulse. *A*, procedure. Block with 300 ms pulses from −50 to +5 mV at 0·33 Hz was followed by an unblocking sequence (90 s at −90 mV, 10 s at −50 mV), n conditioning pulses of duration t (30, 100, 300, 600 or 2400 ms) at 0·33 Hz, and a test pulse 5 s later. *B*, the depression of I_{Ca}(test) − I_{Ca}(block) following n conditioning depolarizations in a muscle treated with 2 μM-D600 for 75 min. Long depolarizations were more effective than short ones. *C*, the decay of I_{Ca} sensitive to block (I_{Ca}(test) − I_{Ca}(block)) as a function of the cumulative time at the conditioning potential ($t \cdot n$). Viewed in this way, short conditioning pulses produce quicker block than long ones.

achieved with 600 and 2400 ms pulses, since inactivation is thought to be very nearly complete within 300 ms at 0 mV (McDonald & Trautwein, 1978*a*). The simplest explanation of the larger block, that D600 interacts with inactivated channels, led us to the next experiment.

Block of inactivated channels. The experimental protocol depicted in the schematic diagram of Fig. 10 was used to examine the association of D600 with inactivated Ca

channels. After a control 300 ms pulse from −50 to +5 mV (C), Ca channels were inactivated by moving the holding potential to +5 mV. One minute later, the control solution was exchanged for one containing 5 μM-D600. After 10 min in the presence of D600, the holding potential was restored to −50 mV and a 300 ms test pulse (D1) was applied 1 min later. The superimposed records of currents triggered by the control and D600 pulses indicate that the drug did not block Ca channels under these experimental conditions. The exposure time to the 5 μM-D600 solution was long enough for a significant block had the muscle been stimulated (see Fig. 2, McDonald *et al.* 1984), and the 1 min rest at −50 mV could not have brought significant unblock (Fig. 4).

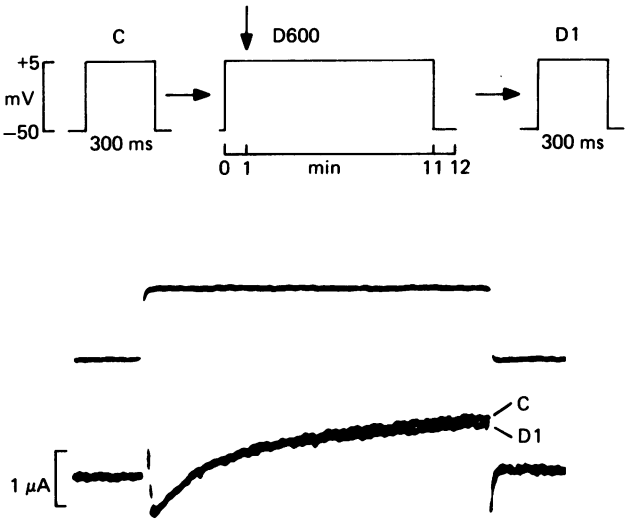

Fig. 10. Inactivated Ca channels are not very susceptible to block by D600. *A*, procedure. After the control 300 ms clamp from −50 to +5 mV (C), the holding potential was moved to +5 mV and superfusion with 5 μM-D600 solution began 1 min later (vertical arrow). Ten minutes later the holding potential was returned to −50 mV and after a 1 min rest, the test pulse (D1) was applied. *B*, the superimposed records show that the drug had very little effect on I_{Ca} under these conditions.

Dependence of the conditioned block on voltage

Between −50 and +10 mV there was a good correspondence between the degree of block and the fraction of Ca channels opened by conditioning pulses (Fig. 8). However, there was an unexplained extra block with conditioning pulses to more positive potentials. Although the size of the extra block between +10 and +50 mV was small relative to the block achieved at +10 mV, there was the possibility that pulses of greater amplitude would have a more potent effect. This was examined in four muscles treated with 2 μM-D600 for 60–90 min, using the protocol shown in Fig. 11*A*. Block with regular stimulation was followed by unblock (90 s, −90 mV), conditioning with two 300 ms pulses to positive potentials V_c, and a standard test pulse from −50 to 0 mV. Both I_{Ca} and the late outward current were measured on the test pulse, normalized with respect to the current on the test following V_c = 0 mV, and plotted against the conditioning potential V_c (Fig. 11*B*). Concentrating first on the I_{Ca} relation (continuous curve), there was a decline from 1·0 at 0 mV to 0·85 near +40 mV, and then an increase to 1·05 at +80 mV. Similar results were obtained in experiments on four other muscles using one or three conditioning pulses.

A plausible explanation for the unexpected shape of the curve in Fig. 11*B* was that the apparent relief of block between +40 and +80 mV was due to a non-specific increase in membrane current subsequent to the unusually large conditioning depolarization. If this were the case, we would also expect corresponding increases in the late outward current (300 ms) on the test pulses. In the two complete experiments documented in Fig. 11*B* (I_{Ca} : ●, ○), the normalized late outward current (dashed line) was independent of V_c. This result argues against the changes in I_{Ca} being related to non-specific after-effects of the large conditioning pulses.

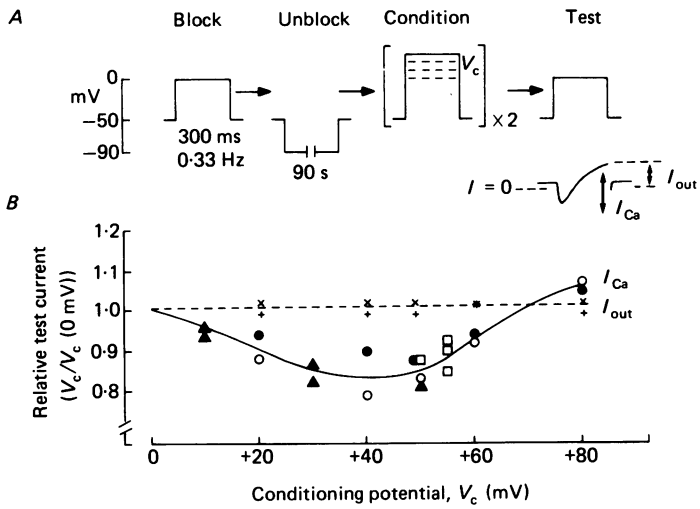

Fig. 11. Dependence of Ca channel block on conditioning pulses to positive potentials in muscles treated with 2 μM-D600 for 60–90 min. *A*, procedure. Ca channels were blocked with regular 0·33 Hz stimulation, unblocked (90 s at −90 mV, 10 s at −50 mV), conditioned with two 300 ms pulses at 0·33 Hz to positive potentials, V_c, and tested for block 10 s later with a 300 ms pulse to 0 mV. Both the outward current at 300 ms (I_{out}) and I_{Ca} were measured on the test pulse. *B*, relative current (test/test after V_c = 0 mV conditioning) *versus* conditioning potential V_c. 'Test' (V_c = 0 mV) was the average of determinations before and after conditioning at another V_c. Plusses and crosses represent I_{out} amplitudes in the two muscles whose I_{Ca} amplitudes are shown as filled and open circles. The remaining symbols represent I_{Ca} from two additional muscles.

Dependence of the time course of the conditioned block on previous conditioning history

Since the time course of block can provide useful information on drug action, we examined whether the time course of the first conditioned block was the same as that of a subsequent conditioned block.

A representative experiment is depicted in Fig. 12. After control stimulation the muscle was clamped to −80 mV and then rested for 20 min in the presence of 2 μM-D600. The first conditioned block was imposed with a set of forty conditioning pulses delivered at 0·33 Hz. Channels were then unblocked during a 20 min rest at −80 mV, and blocked again with a second set of forty conditioning pulses (Fig. 12*A*). I_{Ca} declined by about 70 % during the first conditioning period, was largely restored by the unblocking sequence, but then declined more rapidly during the second conditioning period (Fig. 12*B*). The difference in time course between the first and

second block becomes more obvious when the current on each pulse (n) is normalized ($[I_{Ca}(n)-I_{Ca}(40)]/[I_{Ca}\ (1)-I_{Ca}\ (40)]$) and plotted on a semilogarithmic scale against pulse number (Fig. 12*C*). The time constants, τ, of the fast and slow phases were 2·4 and 11 pulses for the first block, *versus* 1·5 and 8 pulses for the second block. This experiment was repeated on three other muscles but on two of these the unblock of Ca channels was accomplished with 90 s, −90 mV sequences after the initial block. The results were much the same as those in Fig. 12*C*: fast-phase τ values of 2·6–3·5 pulses for the first block *versus* 1·5–2·1 pulses for the second block, and slow-phase τ values of 11·4–15·1 pulses *versus* 8–10·2 pulses.

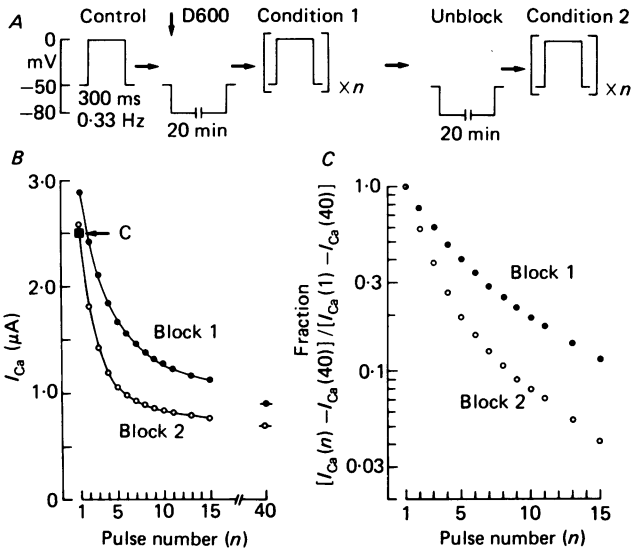

Fig. 12. A comparison of the first conditioned block of I_{Ca} with the second conditioned block. *A*, procedure. Stimulation with 300 ms pulses from −50 to 0 mV at 0·33 Hz (control) was suspended for a 20 min rest period at −80 mV. The control solution was changed to D600 (2 μM) solution at the beginning of this rest. The muscle was then conditioned with forty pulses at 0·33 Hz (condition 1). A second 20 min rest at −80 mV (unblock) was followed by another forty conditioning pulses at 0·33 Hz (condition 2). *B*, I_{Ca} amplitude *versus* conditioning pulse number (n). Blocks 1 and 2 occurred during conditioning 1 and 2; ■ (C) indicates the amplitude of I_{Ca} during control stimulation. *C*, semilogarithmic plot of the time courses of blocks 1 and 2. Both blocks had a fast and slow phase but these were quicker in block 2 than in block 1.

Finally, the influence of the duration and voltage of the unblocking pulse on the subsequent conditioned block was evaluated in six muscles exposed to 2 μM-D600 for 40 min or more. In the example of Fig. 13, conditioning was imposed after unblocks at −50 mV (180 and 600 s) and −90 mV (3–60 s). Even though the amplitude of restored I_{Ca} varied from 2·6 μA (60 s, −90 mV) to 0·7 μA (5 s, −90 mV), the time courses of the subsequent conditioned blocks were all quite similar (Fig. 13*B*). The results on the other five muscles were also consistent with the conclusion that the voltage, duration and degree of unblock have little effect on the time course of the subsequent block.

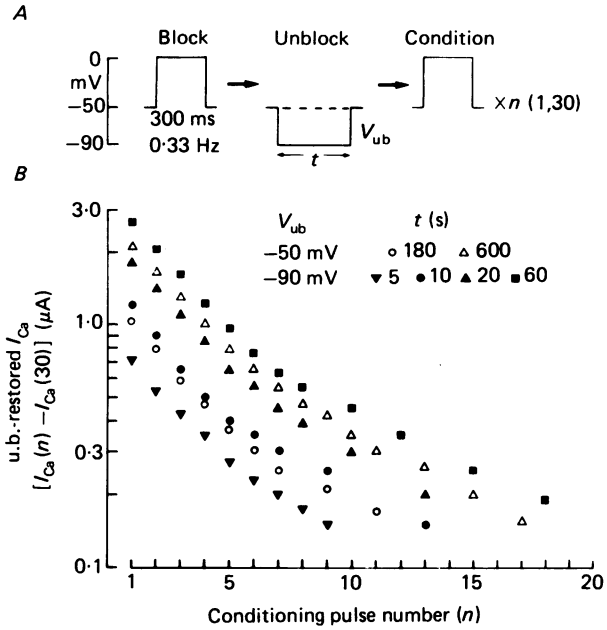

Fig. 13. The dependence of conditioned block development on the degree of the preceding unblock. *A*, procedure. Block with regular stimulation was relieved with an unblocking pulse of duration t to potential V_{ub}; conditioning at 0·33 Hz began 20 s after each unblock. *B*, a semilogarithmic plot of u.b.-restored I_{Ca} *versus* conditioning pulse number in a muscle treated with 2 μM-D600 for 100 min. Unblocking was to a V_{ub} of −50 or −90 mV for the durations denoted by the symbols. The time course of the conditioned block was insensitive to the degree of unblock.

DISCUSSION

The time and voltage dependence of Ca channel block and unblock in D600-treated cat ventricular muscle were examined by varying the frequency, amplitude and duration of voltage-clamp pulses. The results indicate that there is more than a passing resemblance between the interaction of D600 with Ca channels in the heart, and that of local anaesthetics with Na channels in nerve and muscle. In discussing the action of D600, we outline this parallelism and draw on concepts which have evolved from the work on local anaesthetics. The material is organized under these headings: the three components of block; the dependence of conditioned block on channel state; the dependence of conditioned block on voltage; drug–channel interactions leading to conditioned block and unblock; conclusions.

The three components of block

The block of Ca channels by D600 can be separated into three components: the initial, tonic, and conditioned blocks. The initial block occurs in resting tissue equilibrated with the drug. It can be measured only once in a given preparation since its magnitude is indicated by the depression of current on the first post-drug pulse. After a period of stimulation, the tonic component is observed as the block which cannot be removed with a maximally effective unblocking sequence. This leaves the

conditioned block as the difference between tonic block and block induced by depolarizing wave forms.

In D600-treated tissue the initial block was negligible, the tonic block was 15–20 % and the conditioned block was around 75 %. These results are qualitatively similar to those obtained on nerve treated with QX-314 (Strichartz, 1973) or 9-aminoacridine (Yeh, 1979) in that block in the resting membrane is very small, post-rest stimulation greatly increases the block, and tonic block is distinctly larger than initial block. One way of viewing the tonic block is that it is really part of the conditioned block and could perhaps be removed with larger and longer hyperpolarizations. However, in squid axon perfused with QX-312 solution, Cahalan & Almers (1979) found that unlike conditioned block of similar magnitude, tonic block was not accompanied by measurable charge immobilization. In addition, the removal of Na inactivation with pronase abolished the conditioned block but not the tonic block. Thus, it is possible that tonic block occurs by a different mechanism or receptor occupation than conditioned block. However, tonic block may not appear at all, or only very slowly, in the absence of conditioning (Fig. 6). This suggests that access of the drug molecule to a blocking site responsible for tonic block is dependent on membrane depolarization.

The dependence of conditioned block on channel state

Channel state, a key variable in the binding of local anaesthetics to the Na channel (Hille, 1977; Hondeghem & Katzung, 1977), appears to have an important influence on the interaction of D600 with Ca channels. When channels are in the resting state, the binding of D600 is clearly not favoured since conditioned block was relieved at potentials dictating the resting state (i.e. negative to −50 mV). On the other hand, pulsing to channel-opening potentials (positive to −50 mV) led to conditioned block. The rate at which block accumulated was directly related to the frequency of the activating pulses. In addition, the degree of block increased sharply with conditioning potential between −50 and 0 mV, and this dependence resembled the sigmoidal relation between steady-state activation (d_∞) and voltage. These findings suggest that Ca channel openness is a key factor in the binding of D600.

A further indication of the attraction of open Ca channels for D600 was provided by experiments in which conditioning pulse durations ranged from 30 to 2400 ms. Since I_{Ca} inactivation has a time constant of about 70 ms at 0 mV (McDonald & Trautwein, 1978*a*), the average degree of channel openness is greater during a 30 ms pulse than, for example, during a 300 ms pulse. Enhanced binding of the drug to the open channel should manifest itself as a greater block per unit depolarization time with shorter pulses than with longer ones. This was the case, since eight 30 ms pulses (240 ms depolarization) induced a 50 % block while one 300 ms pulse induced a 35 % block. Nevertheless, the fact that a single pulse several seconds in duration produced maximum block, argues for drug binding to inactivated channels as well. Preliminary simulations with a model (in collaboration with J. Šimurda) indicate that a combination of quick binding to open channels and ten times slower binding to inactivated channels provides a suitable explanation of the pulse duration data.

If the binding of D600 to inactivated channels can produce nearly complete block of I_{Ca} within a few seconds (Fig. 9), what is the explanation for the lack of block in

a muscle clamped to +5 mV (channels inactivated) and then exposed to D600 for 10 min (Fig. 10)? The key difference would seem to be that the latter muscle had not undergone any previous conditioning. It may be that repetitive block and unblock provides a route for the translocation of drug from a primary pool in the intracellular fluid to a second pool in the membrane phase. From this staging point, drug may be able to reach the receptor in inactivated channels much faster than can drug from the primary pool. The two-pool concept also helps explain the slow time course of the first conditioned block, and the long times required for steady-state block (McDonald *et al.* 1984) and drug wash-out (Ludwig & Nawrath, 1977; unpublished observations). A further notion is that there may be two binding sites in the channel, one of which can only be reached from the second pool.

In the literature concerning channel state and the block of Na channels by local anaesthetic, there is a heavy emphasis on drug binding to open channels (e.g. Cahalan, Shapiro & Almers, 1980). While this seems to be the major pathway, there is evidence suggesting that interaction of local anaesthetic with inactivated Na channels also leads to block. Khodorov *et al.* (1976) found that a 1 s depolarization produced as much block as a train of 10 ms depolarizations, and Courtney (1981) reported on the 'selective' block of inactivated Na channels in myelinated nerve and skeletal muscle fibres. There is also the phenomenon of conditioned ('extra') block of Na channels in Purkinje fibres exposed to tetrodotoxin (Cohen, Bean, Colatsky & Tsien, 1981). After prolonged rests, single suprathreshold depolarizations of 5–10 s duration produced extra block which developed in two distinct phases. The voltage and time dependence of the fast phase of block resembled channnel activation, and the slow phase was consistent with a binding of toxin to inactivated channels. In that study, and in those of Khodorov *et al.* (1976) and Courtney (1981), strong evidence for drug binding to inactivated channels was obtained by testing for I_{Na} after long conditioning pulses to subthreshold, inactivating potentials. Unfortunately, this protocol could not be employed in the present study because there is a marked overlap of steady-state Ca activation and inactivation in cat ventricular muscle (Trautwein *et al.* 1975; Reuter & Scholz, 1977).

The dependence of conditioned block on voltage

Since the gating of ionic channels is dependent on voltage, it may not always be easy to distinguish between binding dependent on channel state, binding dependent on voltage, and binding dependent on both channel state and voltage. However, the third possibility seems to apply to a large number of local anaesthetics: there is compelling evidence linking channel openness and block, but it is also well established that the curves relating block to conditioning potential lie far to the right of the Na channel activation curve. Very little block is observed at conditioning potentials below 0 mV and the sharpest increase occurs between +20 and +80 mV (Strichartz, 1973; Courtney, 1975; Cahalan, 1978; Cahalan & Almers, 1979; Yeh, 1979). Strichartz (1973) proposed that a drug molecule under the influence of the electric field may have to penetrate some constant distance into the channel before binding can occur (Strichartz, 1973). He also mentioned that the conformation of the binding site might be subject to the membrane potential. Alternatively, a voltage dependence could arise indirectly from voltage-dependent binding of a permeant cation to a site within the channel if both cation and drug compete for the same site (see Woodhull, 1973).

Evidence for the involvement of channel state in the D600 block has already been reviewed. Results which rule out a sole dependence on voltage include the absence of block when a muscle was clamped at +5 mV (Fig. 10), and the presence of two distinct phases in the block produced by long-lasting depolarizations (Fig. 9). A secondary dependence on voltage is possible but far from certain. In contrast to block by local anaesthetics, the relation between D600 block and conditioning potential almost overlies the curve relating Ca channel activation and voltage. However, block apparently unrelated to activation was observed above +10 mV. This extra block was small and reached a peak around +40 mV before declining to zero at +80 mV (Fig. 11). In terms of Strichartz's proposals, it is possible that the favourable influence of a positive electrical field diminishes at strengths above +40 mV, whether by driving the drug beyond the receptor or by reducing the attractiveness of the binding site.

The extra block is difficult to explain on a channel state basis. Between 0 and +40 mV it might be explained by a lengthening of the inactivation time constant (McDonald & Trautwein, 1978*a*). However, a further slowing of inactivation between +40 and +80 mV would not explain the decline of the extra block. Nor can the potential dependence of the extra block be explained by 'Ca-induced inactivation' of Ca channels (Brehm, Eckert & Tillotson, 1980), if indeed this mechanism operates in the heart (see McDonald, 1982; Tsien, 1983). A characteristic feature of Ca-induced inactivation is that 'steady-state inactivation' varies with the amplitude of I_{Ca}: it is maximum near 0 mV (maximum I_{Ca}) and declines with the Ca driving force at more positive potentials. Therefore, in contrast to what is observed, the increasing openness of channels at potentials above 0 mV ought to result in an S-shaped extra block between 0 and +80 mV.

Drug–channel interactions leading to conditioned block and unblock

Pathways which may be important for conditioned block and unblock will be discussed with reference to the channel state diagrams in Fig. 14. In the basic *df*-type model sketched in Fig. 14*A* we make the following assumptions. (a) Drug-free Ca-channels can be found in one of four states: resting (R), open (O), inactivated (I1) (depolarized, activation gates open) and inactivated (I2) (repolarized, activation gates closed). (b) Drug-bound channels can be found in one of the four corresponding states (R*, O*, I1* and I2*). (c) Drug binding and unbinding are not restricted to a particular channel state, although their respective rates may be strongly influenced by channel state. (d) Drug-bound channels may have altered kinetics and equilibria.

Fig. 14*B* illustrates that during depolarization, block could be achieved quickly (heavy arrow) by the association of drug with open channels (O → O*). O* channels would then undergo inactivation to I1*. A much slower block (light arrow) would occur in the case of drug association to inactivated channels (I1 → I1*). Repolarization then promotes the closure of activation gates (I1* → I2*) and eventual restoration of channels to the resting state. In the absence of drug, restoration takes about 0·6 s at −50 mV and 0·25 s at −90 mV (Trautwein *et al.* 1975). Thus, during 0·33 Hz pulsing, drug-bound channels would be in the R* state at the end of the inter-pulse interval unless, as discussed below, drug treatment results in channels getting 'trapped' in the I2* state.

In considering the likely mechanisms of block and unblock, it is useful to begin with

the onset of a conditioned block. After an effective unblocking sequence, I_{Ca} on the first conditioning pulse is large in comparison with I_{Ca} on the subsequent pulse, i.e. block is effected at some point between the onset of the first and second pulses. There are several schemes which could account for this. First, drug molecules may bind to open channels and physically occlude them during the first conditioning depolarization. This removal of channels from the conducting pool might be expected to speed

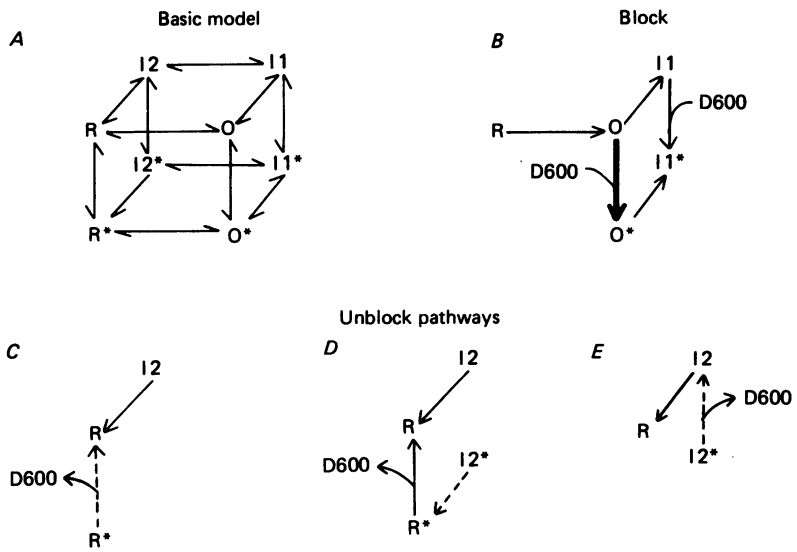

Fig. 14. Drug–channel interaction leading to conditioned block and unblock. *A*, in the basic *df*-type model, drug-free channels have four states: resting (R), open (O), inactivated (I1) (depolarized, activation gates open) and inactivated (I2) (repolarized, activation gates closed). Drug-bound channels have the corresponding states: R*, O*, I1* and I2*. *B*, during depolarization, block is achieved quickly by the association of drug with open channels (heavy arrow) and slowly by association with inactivated channels (light arrow). *C–E*, plausible options for unblock after repolarization to negative potentials. The rate-limiting steps are indicated by the dashed arrows.

up the apparent time course of I_{Ca} inactivation. However, a distinct difference from control time courses was not observed in this study. Therefore, if drug binding to open channels occurs during depolarization, the rate of binding must be slower than the normal rate of inactivation. With the second and succeeding depolarizations in the conditioning train, block would accumulate as more and more channels get shunted into the drug-bound pool mainly via the O → O* pathway. On the assumption that O* channels are physically occluded, it is possible that the removal of inactivation proceeds normally in drug-bound channels, and that unblock occurs by the R* → R pathway (Fig. 14*C*) with unbinding being promoted by hyperpolarizing pulses.

Drug occlusion of open channels is not a mandatory postulate if channels are trapped in the I2* inactivated state after the first conditioning pulse, i.e. closed inactivation gates would be sufficient reason for block on subsequent pulses. An accumulation of drug-bound channels in the I2* state could occur in two ways. First, the rate constant governing the transition I2* → R* may be extremely small so that

effective unblock can only be achieved via a voltage-dependent unbinding step, I2* → I2 (Fig 14*E*). Alternatively, the rate of the I2* → I2 transition may approach zero, leaving a voltage-sensitive I2* → R* step followed by a quick release of drug (R* → R) as the route for unblock (Fig. 14*D*). In other words, a greater hyperpolarization than usual may be required to re-open the gates and facilitate the escape of drug. This concept has been employed to explain the large shift to the left of the h_∞ curve in nerve and muscle exposed to local anaesthetics (Courtney, 1975; Hille, 1977; Schwarz *et al.* 1977; Cahalan *et al.* 1980). An experimental result arguing against D600 causing a real shift of the f_∞ curve is that unblock, though slow, can be achieved at −50 mV. But whether the negative shift of the f_∞ curve in D600-treated muscle reflects an apparent or real shift in the steady-state inactivation, there is no change in the steady-state inactivation of channels not blocked by the drug (Fig. 5). As with local anaesthetics (Schwarz *et al.* 1977), this suggests that D600 acts at a specific receptor rather than by dissolving diffusely in the membrane.

Conclusions

The interaction of D600 with Ca channels in the heart closely resembles the interaction of local anaesthetics with Na channels in nerve and muscle. The common features include: (a) several components of block, (b) accumulation of block with repetitive pulsing, (c) dependence of the degree of block on pulse frequency, amplitude and duration, (d) greater susceptibility to block of open channels than resting or inactivated channels, (e) recovery from block that is slow in comparison with normal recovery from inactivation, and (f) dependence of the rate of unblock on membrane potential. The work on local anaesthetics has had a major impact on our perceptions of Na channel structure and gating, channel receptors, and drug–receptor pathways (Strichartz, 1973; Courtney, 1975; Hille, 1977; Cahalan *et al.* 1980; Khodorov, 1981; Armstrong, 1981). By comparison, little is known about the Ca channel (Hagiwara & Byerly, 1981; McDonald, 1982), and it will be interesting to see whether further studies with Ca channel blockers narrow this gap. On the clinical side, Ca channel blocking agents have become increasingly important in the treatment of cardiac and vascular disorders (Antman, Stone, Muller & Braunwald, 1980). It is almost certain that membrane potential and the frequency of Ca channel opening are key elements in the pharmacology of these drugs. For example, the results of the present study indicate that D600 will exert a stronger blocking action on relatively depolarized areas of the heart (nodal tissue, diseased tissue) than on regions with high resting potential (e.g. ventricle). Finally, it is already clear that physicochemical properties of the drug molecule can influence the interaction of drug with Ca channels (Trautwein *et al.* 1981; Pelzer *et al.* 1982). Broader studies may illuminate drug–receptor pathways and contribute to the design of clinically useful agents.

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